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Phorbol Esters Modulate the Phosphorylation of Human T-Cell Leukemia Virus Type I Tax

JOSEPH D. FONTES,[1]† JUDITH M. STRAWHECKER,[1] NATHAN D. BILLS,[1] ROBERT E. LEWIS,[2,3] AND STEVEN H. HINRICHS[1,3]*

Departments of Pathology and Microbiology[1] and Biochemistry and Molecular Biology[2] and The Eppley Institute for Research in Cancer and Allied Diseases,[3] University of Nebraska Medical Center, Omaha, Nebraska 68198-6495

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The Tax protein from human T-cell leukemia virus type I (HTLV-I) is a 40-kDa phosphoprotein capable of activating transcription from its own long terminal repeat (LTR), as well as increasing the transcription of cellular genes. Transcriptional activation of the HTLV-I LTR has been demonstrated via a cyclic-AMP-responsive element within the 21-bp Tax-responsive elements of the LTR. Phorbol esters also upregulate expression via the LTR. Since phosphorylation of Tax may play a role in these processes, we investigated the relative effects of kinase-stimulating agents on ^{32}P incorporation into Tax. Our studies demonstrated that the phorbol ester 4β-phorbol-12β-myristate-13α-acetate greatly stimulated Tax phosphorylation in a time- and dose-dependent manner. In contrast, 8-bromoadenosine 3′-5′-cyclic monophosphate induced little stimulation of Tax phosphorylation. Tax phosphorylation occurred only on serine residues and was mapped to a single tryptic fragment in both Tax-producing human lymphocytes and mouse fibroblast cells.

The Tax protein from human T-cell leukemia virus type I (HTLV-I) is a strong transcriptional activator of its own long terminal repeat (LTR) (32, 35). HTLV-I is the etiologic agent of proliferative, as well as degenerative, phenomena in infected humans. It causes a rapidly fatal T-cell malignancy termed adult T-lymphocytic leukemia (ATL) (8) and the neurodegenerative disease tropical spastic paraparesis–HTLV-I-associated myelopathy (9, 26). Little is known about the pathogenesis of these conditions, but a great deal of inquiry has centered on the *trans*-acting factor Tax and its effect on an expanding list of cellular genes, many of which are involved in regulation of cell proliferation (3, 6, 7, 22, 37). Current hypotheses regarding HTLV-I-driven transformation focus on the disruption of normal cellular gene regulation by the Tax protein (33).

Tax is known to be a phosphoprotein composed of 353 amino acids. It is localized principally in the nucleus, as its function suggests (35). Mutation studies of Tax have identified specific functional regions. The first 50 amino acids contain a nuclear localization signal, and the distal carboxy terminus is dispensable for transactivation function (31, 34). Also, within the first 50 amino acids there is a cysteine-rich region with zinc-binding activity (30).

Nyunoya et al. demonstrated that the protein is phosphorylated on one or more serine residues when it is expressed in BmN insect cells with a baculovirus expression system (25). It is unclear whether phosphorylation of Tax is relevant to its role in mammalian eukaryotes. No reports have described the effect of phosphorylation on Tax protein in a Tax-transformed cell line. The role of phosphorylation in transcriptional regulation is a subject of general interest and has been demonstrated for cellular transcriptional factors, such as some members of the CREB-ATF family (20, 38). Phosphorylation has been shown to play a role in regulation of both cellular and viral genes and may alter binding or cellular trafficking of several viral genes, including simian virus 40 T antigen, adenovirus E1A, and HTLV-I Rex (14). Phosphorylation of Rex has been shown to result in the accumulation of unspliced viral mRNA (2).

Although much has been learned regarding the regulation of HTLV-I, it remains to be determined why leukemia or lymphoma develops in such a low percentage of individuals infected with the virus. Evidence for a direct role in transformation by the Tax protein was obtained in transgenic mouse experiments in which Tax was expressed under the regulation of its own LTR (23). The development of ATL in less than 1% of infected humans is in stark contrast to findings in transgenic experiments in which all animals developed tumors with a predictable and consistent time of onset (13). It has also been shown that transformation in the HTLV-I Tax transgenic system is cell type specific and neither lymphocytes nor muscle cells expressing Tax become transformed (24). It is not known whether phosphorylation is responsible for the different cell type and species effects of Tax. To address this question, we chose to identify agents capable of stimulating phosphorylation and map the site(s) of phosphorylation within Tax.

Phorbol esters, okadaic acid (OA), and serum enhance ^{32}P incorporation into Tax in Px-1 cells. It has been previously demonstrated that transcriptional activation of the HTLV-I LTR by cyclic AMP occurs via the cyclic-AMP-responsive element within the 21-bp Tax-responsive elements of the LTR (15, 27). The mechanism for this effect may be through cyclic AMP activation of protein kinase A (PKA) (29). Expression from the LTR is also upregulated by phorbol esters which are protein kinase C (PKC)-activating agents (15, 28). Since phosphorylation of Tax may play a role in these processes, we tested the abilities of agents which activate PKA or PKC pathways to stimulate phosphorylation of Tax in cells derived from transgenic mouse tumors expressing Tax. One such cell line, termed Px-1, was used for these studies. After Px-1 cells were metabolically labeled in vitro with $^{32}P_i$, the Tax protein was immunoprecipitated

* Corresponding author.
† Present address: Howard Hughes Medical Institute, University of California at San Francisco, San Francisco, CA.

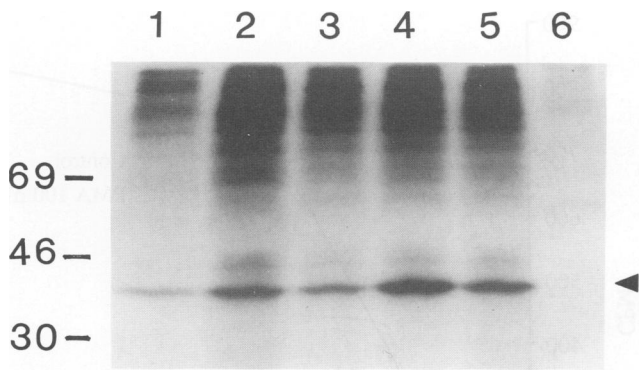

FIG. 1. Detection of HTLV-I Tax in Px-1 cells labelled with ^{32}P. Px-1 cells expressing Tax were plated on 35-mm-diameter plastic culture dishes, grown until confluent, and incubated for 2 h in phosphate-free Dulbecco modified Eagle medium at 37°C; $^{32}P_i$ (New England Nuclear) was added to a final concentration of 2 mCi/ml; and incubation was continued for 2 h. Cells received either no treatment (lanes 1 and 6) or addition of 10 nM OA (lane 2), 0.5 mM 8-Br-cAMP (lane 3), 1 μM PMA (lane 4), or 10% fetal bovine serum (lane 5) for an additional 30 min, after which the reaction was terminated by aspiration of the media and placement of the cell culture plate on liquid N_2. Immunoprecipitations were performed with a polyclonal antibody against HTLV-I Tax (no. 467, provided by the National Institutes of Health AIDS resources repository) (lanes 1 to 5) or nonimmune serum (lane 6), and then protein A-Sepharose was added (19). Tax phosphorylation was quantitated by counting the excised bands on a scintillation counter. Protein markers are indicated at the left (molecular sizes are in kilodaltons), and Tax is identified with an arrowhead.

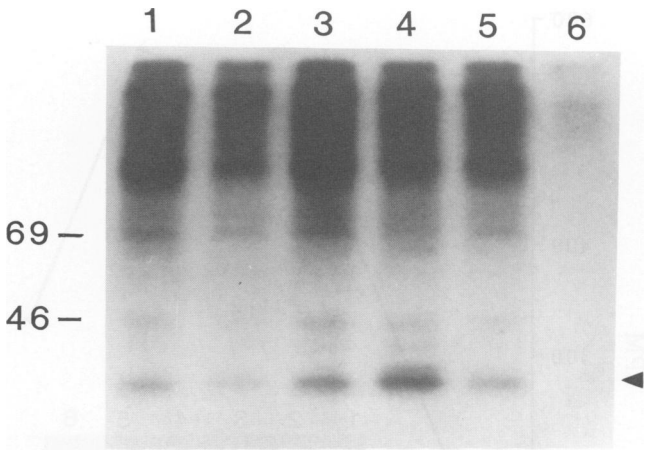

FIG. 2. Detection of HTLV-I Tax in MT-4 cells labelled with ^{32}P. MT-4 cells expressing Tax were incubated for 2 h in phosphate-free Dulbecco modified Eagle medium at 37°C, and then $^{32}P_i$ was added. Cells received either no treatment (lanes 1 and 6) or addition of 10 nM OA (lane 2), 0.5 mM 8-Br-cAMP (lane 3), 1 μM PMA (lane 4), or 10% fetal bovine serum (lane 5) for an additional 30 min, after which the reaction was terminated. Immunoprecipitations were performed with a polyclonal antibody against HTLV-I Tax (no. 467) (lanes 1 to 5) or nonimmune serum (lane 6), and then protein A-Sepharose was added (19). Tax phosphorylation was quantitated by counting the excised bands on a scintillation counter. Protein markers are indicated at the left (molecular sizes are in kilodaltons), and Tax is identified with an arrowhead.

with a polyclonal antibody and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in accordance with established procedures (18). Autoradiographs of the dried gel demonstrated the 40-kDa Tax protein (Fig. 1, lane 1). Substitution of nonimmune serum for anti-Tax serum led to loss of the 40-kDa band (Fig. 1, lane 6). Addition of 0.5 mM 8-bromoadenosine 3′-5′-cyclic monophosphate (8-Br-cAMP) (lane 3) and 1 μM 4β-phorbol-12β-myristate-13α-acetate (PMA) (lane 4) to the culture media for 30 min prior to harvest stimulated incorporation of ^{32}P into the Tax protein compared with ^{32}P incorporation into untreated cells (lane 1). We also studied the effect of 10% fetal bovine serum and OA, a specific inhibitor of phosphatases 1 and 2A (12), on Tax phosphorylation. Px-1 cells incubated with $^{32}P_i$ in the presence of 10 nM OA or 10% fetal bovine serum also demonstrated an increase in phosphorylation levels of Tax (lanes 2 and 5) in comparison with untreated cells (lane 1). The effect of PMA stimulation on Tax phosphorylation (approximately fourfold) was quantitated by scintillation counting of the excised radiolabelled bands. Intermediate effects (twofold) were seen with addition of serum or OA to Px-1 cell cultures. 8-Br-cAMP had little effect on the phosphorylation of Tax.

MT-4 and Px-1 cells respond similarly to PMA. Differences in the site and degree of phosphorylation between transformed human cells and cells from the transgenic system may account for differences in neoplastic transformation. The MT-4 cell line produces functional Tax as determined by *trans*-activation assays but has no detectable reverse transcriptase activity (21). To determine whether human cells transformed by HTLV-I respond to PMA similarly to Px-1 cells, we metabolically labelled MT-4 cells as described above for Px-1 cells. As seen in Fig. 2, PMA again induced a significant increase in Tax phosphorylation in MT-4 cells above controls in comparison with the other agents. We noted a biochemical advantage the Px-1 cells provided in that absolute levels of phosphorylated Tax in response to PMA were greater in Px-1 than in MT-4 cells, as shown in Fig. 3, where total immunoprecipitate from extracts of 10^6 Px-1 cells gave a more intense band than 10^7 MT-4 cells.

Stimulation of Tax phosphorylation by PMA occurs within 60 min. To optimize conditions for a dose-response study of the effect of PMA on Tax phosphorylation, a preliminary experiment was performed with stimulation for either 15 or 30 min with a range of PMA doses (data not shown).

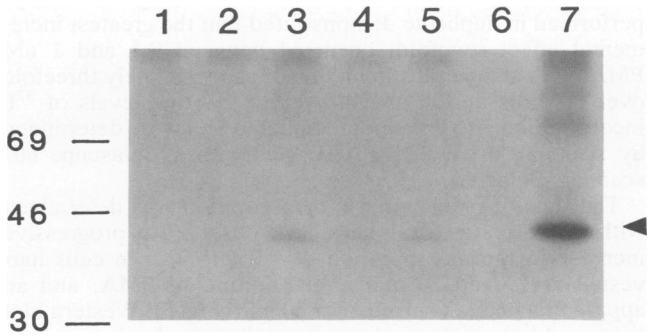

FIG. 3. Stimulation of HTLV-I Tax phosphorylation in MT-4 cells compared with Px-1 cells. MT-4 (lanes 1 to 5) and Px-1 (lane 7) cells were treated with standard concentrations of PMA for 30 min after incubation in phosphate-free media and $^{32}P_i$. Tax protein was immunoprecipitated as described in the legend to Fig. 1. Lanes: 1 and 2, no treatment; 3, 10 nM PMA; 4, 50 nM PMA; 5 and 7 (Px-1 cells), 100 nM PMA. Lane 6 was blank. The numbers at the left are molecular sizes in kilodaltons.

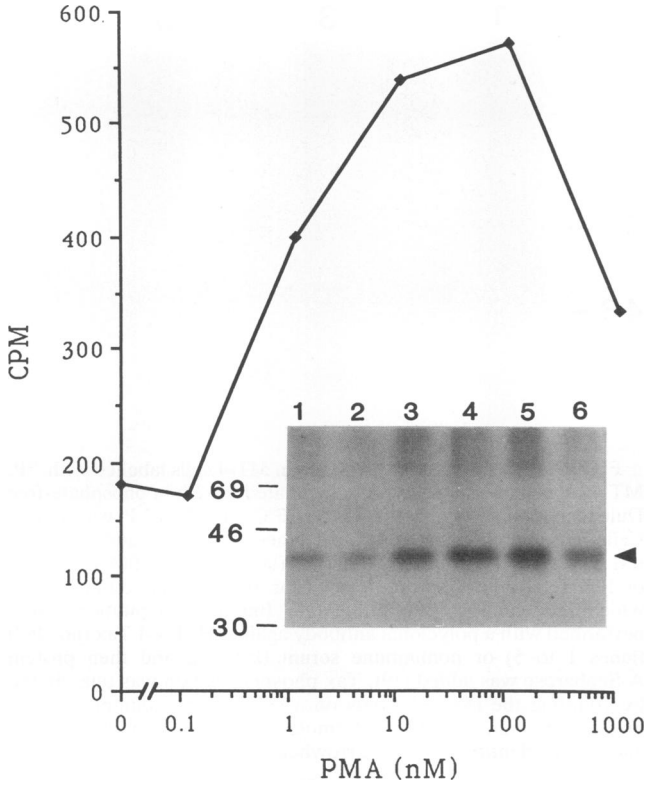

FIG. 4. PMA dose response of Tax phosphorylation. Px-1 cells were incubated with ^{32}P as before, and 0.1, 1, 10, 100, and 1,000 nM concentrations of PMA were added for 60 min, after which the reactions were terminated and Tax was immunoprecipitated. Experiments were run in duplicate. After electrophoresis, the dried gels were counted on a Betagen Betascope 603 Analyzer and counts per minute (CPM) were quantitated from each of the Tax protein bands and plotted. The inset is an autoradiograph of the gel containing immunoprecipitated Tax from Px-1 cell cultures without treatment (lane 1) and those containing PMA (lanes: 2, 0.1 nM; 3, 1 nM; 4, 10 nM; 5, 100 nM; 6, 1000 nM). Arrowhead, Tax. The numbers to the left of the inset indicate molecular sizes in kilodaltons.

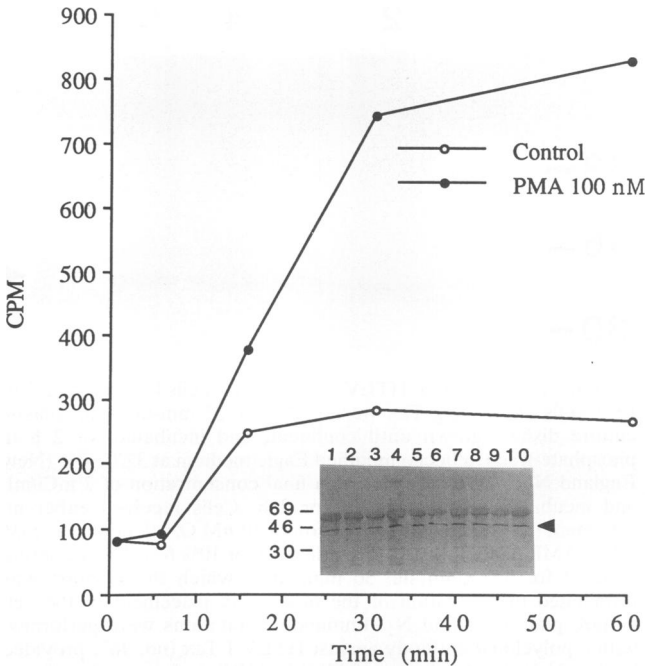

FIG. 5. Time course of Tax phosphorylation in response to PMA stimulation. Px-1 cells were labelled with ^{32}P as before, in the presence (●) or absence (○) of 100 nM PMA for 0, 5, 15, 30, or 60 min. Immunoprecipitation and electrophoresis were performed as before, and gels were analyzed with a Betagen Betascope. CPM, counts per minute. The inset is a Western blot of nonradiolabelled, immunoprecipitated Tax treated as described above. Supernatants were subjected to SDS–12% PAGE (18) and transblotted to Hybond-C membranes (Amersham) (36). Tax bands were localized with monoclonal antibody 168B17 and developed with a blotting detection kit for mouse antibodies (Amersham RPN 22). Odd-numbered samples were without PMA treatment, and even-numbered samples were treated with 100 nM PMA. Lanes: 1 and 2, 0 min; 3 and 4, 5 min; 5 and 6, 15 min; 7 and 8, 30 min; 9 and 10, 60 min. The arrowhead indicates the 40-kDa Tax protein. The diffuse band above Tax is the immunoglobulin heavy chain. The numbers to the left of the inset indicate molecular sizes in kilodaltons.

Dose-dependent phosphorylation of Tax by PMA was demonstrated by addition of 0 to 1,000 nM PMA to Px-1 cells in ^{32}P-containing media for 60 min at 37°C (Fig. 4). Assays performed in duplicate demonstrated that the greatest incremental effect (twofold) occurred between 0.1 and 1 nM PMA, with an overall stimulation of approximately threefold over controls at 100 nM PMA. The relative levels of ^{32}P incorporation into immunoprecipitated Tax were determined by scanning dried SDS-PAGE gels with a Betascope 603 scanner (Betagen).

The time of maximum ^{32}P incorporation was determined with PMA-treated Px-1 cells. We observed a progressive increase in the incorporation of ^{32}P into Tax in cells harvested at 15 and 30 min after addition of PMA, and an apparent plateau occurred after 60 min (Fig. 5). Western blot (immunoblot) analysis demonstrated that the increase in phosphorylation was not due to a PMA-induced increase in Tax synthesis, since equivalent levels of Tax were present at each time point (Fig. 5, inset). A similar experiment, also performed with PMA-treated MT-4 cells, demonstrated that Tax protein levels remained constant over the same time period (data not shown). Stimulation of phosphorylation has also been described for HTLV-I Rex after treatment of HTLV-I-infected cells with 12-*O*-tetradecanoylphorbol-13-acetate for 15 min (1). Like that of Rex, the subcellular location where Tax is phosphorylated is unknown. Activation of PKC by phorbol ester occurs through translocation of the cytostolic kinase to a membrane-bound form. Following interleukin 4-induced activation of monocytes, 69% of total PKC activity is found in nuclear fractions (4). However, the subcellular distribution of PKC in Tax-expressing cells and whether it is the direct kinase involved have not been determined. These findings emphasize the importance of identifying the subcellular site and the kinase involved for phosphorylation of HTLV-I regulatory proteins.

The Tax protein is phosphorylated on serine. To identify which amino acids are phosphorylated in the Tax protein, the 40-kDa bands from the SDS-PAGE gel of immunoprecipitated, ^{32}P-labelled Tax were excised and acid hydrolyzed. The hydrolysates were analyzed by two-dimensional thin-layer chromatography (TLC) and compared with phosphoserine, phosphothreonine, and phosphotyrosine amino acid standards (Fig. 6A). Only phosphoserine was detected, regardless of whether cells were untreated or treated with 8-Br-cAMP, OA, PMA, or serum (data not shown).

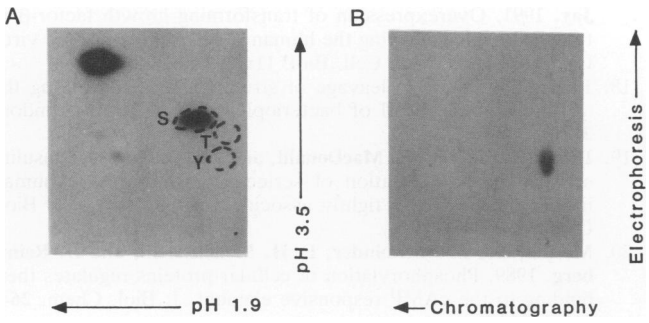

FIG. 6. (A) Two-dimensional TLC phosphopeptide analysis of ^{32}P-labelled, immunoprecipitated Tax protein. ^{32}P-labelled Tax protein was eluted by the method of Boyle et al. (5), and phosphoamino acid analysis was performed by high-voltage electrophoresis and autoradiography as previously described (19). S, phosphoserine; T, phosphothreonine; Y, phosphotyrosine. The unidentified spot in the upper left corner is free phosphate. (B) Two-dimensional TLC analysis of ^{32}P-labelled TPCK-trypsin phosphopeptide fragments. ^{32}P-labelled Tax immunoprecipitated from Px-1 cells was eluted and digested with TPCK-treated trypsin as previously described (19). The phosphopeptide fragments were resolved in two dimensions by electrophoresis and chromatography on cellulose thin-layer plates. Origins were in the bottom right corners. After chromatography, the plates were dried and exposed to X-ray film to localize ^{32}P-labelled peptides.

Tax phosphorylation is limited to a single tryptic fragment. To determine the approximate site of the phosphorylated serine(s) within Tax, the 40-kDa bands from SDS-PAGE gels of labelled Tax from treated and untreated cells were excised and digested with tolylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-trypsin. The digests were resolved by two-dimensional TLC, and a single fragment was identified by autoradiography (Fig. 6B). Treatment of Px-1 cells with PMA, 8-Br-cAMP, or OA increased the intensity of labelled fragments but did not result in any additional phosphopeptides. Consensus amino acid sequences in Tax for PKC were sought by comparison and alignment of published phosphorylation sites for a number of proteins (16). Four motifs that match common PKA or PKC phosphorylation sites were identified as likely candidates for phosphorylation of the Tax protein in Px-1 cells. Three of these sites are within the amino-terminal one-third of the protein and reside on separate tryptic fragments (Table 1).

To determine whether the site of Tax phosphorylation differed between the human MT-4 cells and the transgenic mouse tumor Px-1 cell line, two-dimensional TLC was performed on tryptic digests of Tax from PMA-treated MT-4 and PX-1 cells. Autoradiography showed that Tax from either cell type yielded labelled tryptic fragments identical in mobility (Fig. 7).

TABLE 1. Serine phosphorylation consensus sites in Tax

Amino acid[a] at minimal PKA-PKC consensus site:					
1	2	3	4	5	6
X	R	X	S		
R	X	X	S		
			S	X	R
G	L	C	S-37	A	R
R	L	P	S-77	F	P
Q	R	T	S-84	K	T
R	K	Y	S-113	P	F
G	L	C	S-334	A	R

[a] Consensus phosphorylation sequences are shown above the gap, and potential serine Tax phosphorylation sites are shown below the gap. X, any amino acid.

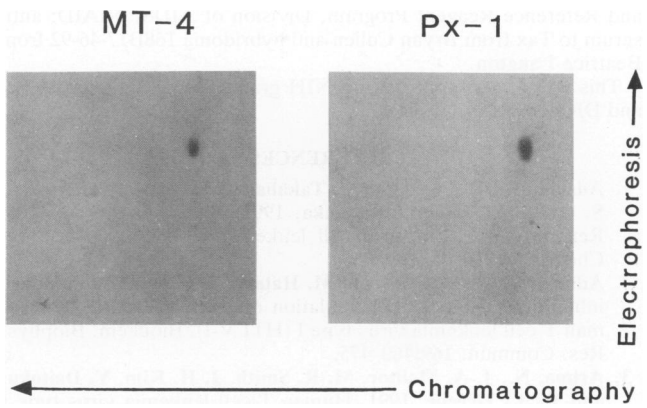

FIG. 7. Tryptic fragments of phosphorylated Tax from MT-4 and Px-1 are identical in migration. Tax protein was labelled, immunoprecipitated, and eluted as described in the previous figure legends. Two-dimensional TLC tryptic digest fragment analysis was performed as described in the legend to Fig. 6B.

LTR-*tax* transgenic mice (23) provide an excellent model for dissection of the molecular events that lead to Tax-driven tumorigenesis. Tumors from these mice can be disrupted and grown in culture, and several cell lines have been obtained. Cell lines derived from these mice have been extensively studied with respect to cellular genes that are transactivated by Tax (10, 11, 17). We observed by Western blotting approximately five times more Tax in Px-1 cells than in MT-4 cells when comparable amounts of total protein were used (data not shown). In experiments studying the phosphorylation of HTLV-I Rex, it was found that similar levels of Rex were present in two human T-cell lines and one rat T-cell line (1). Our interest is in determining whether levels of Tax expression correlate with the rapidity of progression to neoplasia.

The level of induction of Tax phosphorylation seen in our studies was significantly affected by PKC-acting agents but not by PKA-acting agents. OA, an inhibitor of phosphatases 1 and 2A, also led to an increase in Tax phosphorylation in Px-1 cells that was not observed in MT-4 cells. No additional phosphorylation sites were induced by any of the agents tested. Future studies will investigate further the rate of phosphorylated Tax turnover and the specific kinases involved. The role of Tax phosphorylation in pathogenesis remains unclear, since bacterially synthesized Tax (nonphosphorylated) is capable of inducing transactivation of its own LTR in vitro (39). However, Tax serine mutants lose the ability to transactivate the human immunodeficiency virus LTR (31). It is possible that phosphorylation of Tax may affect the specific activity of the protein and not its absolute function. Identification of the exact site of phosphorylation will allow study of the function of Tax phosphorylation through mutation studies.

J. Fontes and J. Strawhecker contributed equally to this work. We thank Dee Volle for technical assistance and Pat Koso for secretarial assistance.

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